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Whole-cell uptake and nuclear localization of 1,25-dihydroxycholecalciferol by breast cancer cells (T47 D) in culture

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Specific high-affinity receptors for 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$] have been described recently in broken-cell preparations of several cultured human breast cancer cell lines including the T47 D line. It was necessary to determine whether intact breast cancer cells in culture would bind 1,25-$(OH)_2D_3$ specifically and whether the next step in the proposed scheme of action, i.e. nuclear translocation, occurred. The following results were obtained. (1) Specific uptake of 1,25-$(OH)_2D_3$ by T47 D cells occurs in intact cells in culture. (2) The rate of uptake is proportional to medium 1,25-$(OH)_2D_3$ concentration but is slow compared with that of other steroid hormones, e.g., oestradiol, under identical conditions. Even at 0.5 nM-1,25-$(OH)_2D_3$ in the medium, at least 4 h are required to reach maximum compared with less than 1 h for oestradiol binding. (3) Estimation of binding characteristics by Scatchard analysis indicates a single class of binding sites with K_d of 68 pM and 11 800 binding sites/cell, which are similar results to those obtained with broken-cell preparations. (4) Inclusion of various vitamin D metabolites in the incubation medium decreased specific binding of 1,25-$(OH)_2D_3$ by the intact cells in a manner identical with their effects in the broken-cell preparation and with potencies similar to their potency on Ca^{2+} transport and bone resorption *in vivo*. Order of potency was 1,25-$(OH)_2D_3$ > (24*R*)-1,24,25-trihydroxycholecalciferol ≫ 25-hydroxycholecalciferol > (25*R*)-24,25-dihydroxycholecalciferol ≫ (25*R*)-25,26-dihydroxycholecalciferol. (5) In the 1,25-$(OH)_2D_3$-depleted state, 80% of the 1,25$(OH)_2D_3$ receptor is found in the cytosol fraction of the cells even when the subcellular fractionation is performed under low-salt conditions. By contrast after incubation with [^{3}H]1,25-$(OH)_2D_3$, 59% of the specific 1,25-$(OH)_2D_3$ binding is found in the partially purified nuclei fraction. These data indicate that nuclear translocation of the receptor–hormone complex takes place in the intact T47 D cell. The results also support the hypothesis that the 1,25-$(OH)_2D_3$ receptor is functional in this cultured breast cancer cell line, which may provide a useful model for further study of the early biochemical events in 1,25-$(OH)_2D_3$ action.

Vitamin D action is thought to be mediated largely through its dihydroxylated metabolite 1,25-$(OH)_2D_3$ in a manner analogous to that of the steroid hormones (DeLuca, 1979; Haussler & McCain, 1977; Norman, 1980). This hypothesis is supported by two major pieces of evidence. First, high-affinity specific cytosolic receptors for 1,25-$(OH)_2D_3$ have been demonstrated in its well characterized target organs, i.e. intestine (Tsai & Norman, 1973; Brumbaugh & Haussler, 1974; Kream *et al.*, 1976; Kream & DeLuca, 1977) and bone (Kream *et al.*, 1977; Manolagas *et al.*, 1979; Chen *et al.*, 1979). Secondly, a steroid-like mechanism is supported by the demonstration of nuclear localization of hormone *in vivo* (Stumpf *et al.*, 1979, 1980) and association of hormone–receptor complexes with nuclear components *in vitro* (Brumbaugh & Haussler, 1974; Colston & Feldman, 1980; Walters

Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2D_3$, (25*R*)-24,25-dihydroxycholecalciferol; 25-$(OH)D_3$, 25-hydroxycholecalciferol; 1,24,25-$(OH)_3D_3$, (24*R*)-1,24,25-trihydroxycholecalciferol; 25,26-$(OH)_2D_3$, (25*R*)-25,26-dihydroxycholecalciferol.

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et al., 1980) despite the fact that the specific gene product(s) responsible for Ca^{2+} transport has not been conclusively identified. On the basis of this hypothetical scheme the presence of 1,25-$(OH)_2D_3$ receptor has been taken to indicate a 1,25-$(OH)_2D_3$-responsive organ, particularly where calcium phosphate transport or regulation is involved, e.g., kidney, oviduct and parathyroid (Brumbaugh *et al.*, 1975; Christakos & Norman, 1979; Colston & Feldman, 1979; Coty, 1980).

We have recently demonstrated that high-affinity specific receptors for 1,25-$(OH)_2D_3$ are present in several cultured breast cancer cell lines as well as in normal functional rabbit breast tissue (Eisman *et al.*, 1979, 1980*a*,*b*,*c*; Martin *et al.*, 1980; Findlay *et al.*, 1980). Receptors are also present in a high proportion of human primary breast cancers, but are uncommon in other malignancies (E. Sher, J. A. Eisman, J. M. Moseley & T. J. Martin, unpublished work). The experiments reported here demonstrate time-dependent specific uptake of [^{3}H]1,25-$(OH)_2D_3$ by the cultured breast cancer cell line T47 D and nuclear translocation of the hormone.

Materials and methods

T47 D cells were supplied to Dr. R. Whitehead by the Breast Cancer Task Force, Washington, DC, U.S.A. 1,25-Dihydroxy[23,24-^{3}H]cholecalciferol (sp. radioactivity 102 Ci/mmol) and 25-hydroxy-[26,27-^{3}H]cholecalciferol (sp. radioactivity 22 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled 25-$(OH)D_3$, 24,25-$(OH)_2D_3$, 1,24,25-$(OH)_3D_3$, 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were gifts from Dr. M. Uskoković, Hoffman–La Roche, Nutley, NJ, U.S.A. Roswell Park Memorial Institute ('RPMI') 1640 medium was obtained from Flow Laboratories Australasia, Armidale, N.S.W., Australia, foetal-calf serum from Grand Island Biological Co. Biocult, and phosphate-buffered saline from Oxoid, London, U.K. Costar tissue-culture plates were obtained from Disposable Products, Cambridge, MA, U.S.A. Instagel was obtained from the Packard Instrument Co., Downers Grove, IL, U.S.A. Other chemicals were of highest grade available from standard suppliers.

Cell culture

For routine maintenance T47 D cells were cultured in RPMI 1640 medium containing 5% (v/v) foetal-calf serum to which was added 0.1 μM-insulin (Novo porcine) and 0.1 μM-cortisol hemisuccinate. The latter hormones were omitted from the time of final subculture for the 2–5 days before use in experiments. Normal foetal-calf serum was replaced with dextran/charcoal-treated foetal-calf serum (Lippman & Bolan, 1975) for 24–48h before experiments. Cells were confluent at the time of experiments at a density of $(3.7 \pm 0.1) \times 10^5$ cells/cm^2.

Measurement of 1,25-$(OH)_2D_3$ uptake

Intact-cell uptake studies were carried out with cells grown in 12- or 24-well multi-well Costar plates. [^{3}H]1,25-$(OH)_2D_3$ at various concentrations was added in RPMI 1640 medium with 0.05% bovine serum albumin with or without a 100-fold molar excess of unlabelled 1,25-$(OH)_2D_3$ into replicate wells. After various periods of incubation at 37°C up to 24h, the medium was removed, and the cells were rinsed twice with 0.5% bovine serum albumin in phosphate-buffered saline (Oxoid), and twice with phosphate-buffered saline alone. The cells were allowed to equilibrate with the first bovine serum albumin-containing phosphate-buffered-saline rinse for 15–20min at 20°C, as this decreased non-specific binding markedly (results not shown). After the final phosphate-buffered-saline wash the cells were removed with 1.0M-NaOH (0.5ml/cm^2 per well), transferred to a scintillation vial, neutralized with acetic acid and counted for radioactivity using Instagel scintillation fluid. In some experiments uptake of [^{3}H]1,25-$(OH)_2D_3$ (70pM) was examined in media where the bovine serum albumin was replaced with 5% charcoal-treated foetal-calf serum with and without unlabelled 25nM-25-$(OH)D_3$. In other experiments the uptake of [^{3}H]1,25-$(OH)_2D_3$ (250pM) was examined after 6h incubation in medium containing 0.05% bovine serum albumin and various concentrations of either unlabelled 1,25-$(OH)_2D_3$ or other cholecalciferol metabolites. The uptake of 25-$(OH)D_3$ was examined in the cultured cells under similar conditions, i.e. [^{3}H]25-$(OH)D_3$ (25nM) with and without a 250-fold molar excess of unlabelled 25-$(OH)D_3$ was added to the cells in RPMI 1640 medium supplemented with 0.05% bovine serum albumin and incubated for $\frac{1}{2}$ to 6h.

Scatchard analysis of the uptake of [^{3}H]1,25-$(OH)_2D_3$ was performed on the data obtained after 10–12h incubation when binding was maximal. To study the egress of 1,25-$(OH)_2D_3$ from these cells, cells were pre-incubated at 37°C with [^{3}H]1,25-$(OH)_2D_3$ (250pM) with or without unlabelled 1,25-$(OH)_2D_3$ in RPMI 1640 medium containing 5% charcoal-treated foetal-calf serum. After 16h this medium was removed and replaced with fresh 1,25-$(OH)_2D_3$-free medium. After various times of further incubation up to 48h at 37°C, the cells were harvested and counted for radioactivity as described above.

Subcellular localization of receptor

For subcellular localization studies, cells grown in 75cm^2 flasks were incubated with [^{3}H]1,25-$(OH)_2D_3$

(100 pM) with or without unlabelled 1,25-$(OH)_2D_3$. After various times the cells were rinsed as above, and removed with a rubber policeman. Disruption of cells was achieved by hypo-osmotic lysis in buffer A for 30 min at 4°C and passage through a 23-gauge stainless-steel needle. Buffer A contained 1.5 mM-$CaCl_2$ in 5 mM-imidazole, pH 7.4. Fractionation of the cells into various subcellular components (Marver & Edelman, 1975) was as follows. The supernatant fraction from centrifugation at 1000 *g* for 10 min was further centrifuged at 100000 *g* for 1 h to provide a supernatant or cytosol fraction and a pellet consisting largely of mitochondria, microsomes and other endoplasmic-reticulum components. The initial low-speed pellet was suspended in buffer B, which contained 1.5 mM-$CaCl_2$, 2 mM-dithiothreitol, 20 mM-imidazole (pH 7.4), 0.25 M-sucrose and 5000 i.u. of aprotinin/ml. This crude nuclear pellet was recentrifuged, suspended in 2 ml of 2.2 M-sucrose in buffer B and layered on top of a cushion of 1.6 ml of 2.2 M-sucrose in buffer B. After centrifugation at 300000 *g* (Beckman SW 50.1 rotor at 45000 rev./min and 8.35 $r_{av.}$) for 20 min the partially purified nuclei, which pelleted at the bottom of the tube, were extracted with 0.1 M- and/or 0.4 M-KCl in buffer B. In the experiments where the cells had been incubated with [^{3}H]1,25-$(OH)_2D_3$ the individual fractions were corrected for variations in efficiency by the use of internal standards and the external-standard ratio. In parallel experiments, cells incubated under identical conditions but without [^{3}H]-1,25-$(OH)_2D_3$ were fractionated identically. Subsequently these fractions were brought to 0.1 M-KCl in buffer B before incubation at 4°C with 50 pM-[^{3}H]1,25-$(OH)_2D_3$ with and without a 100-fold molar excess of unlabelled 1,25-$(OH)_2D_3$. Incubations were at 25°C for 2 h and at 4°C for 4 h. Separation of bound from free ligand was achieved by using 0.1% dextran T70/1.0% charcoal (0.2 ml/ml of incubation mixture). After 30 min incubation at 4°C and centrifugation at 2000 *g* for 15 min at 4°C, the bound hormone in the supernatant was counted with Instagel scintillation fluid. Recovery of nuclei was estimated from recovery of DNA in cells purified in parallel with the experimental cells (Setaro & Morley, 1976).

Results

The T47 D cells growing in monolayer culture exhibited specific uptake of [^{3}H]1,25-$(OH)_2D_3$ with a prolonged time course reaching maximum binding after at least 8 h (Fig. 1). The rate of uptake and final specific binding achieved were both decreased in the presence of foetal-calf serum. However, the omission of any protein from the incubation medium resulted in unacceptable losses of [^{3}H]1,25-$(OH)_2D_3$ on to glassware and culture dishes. The addition of

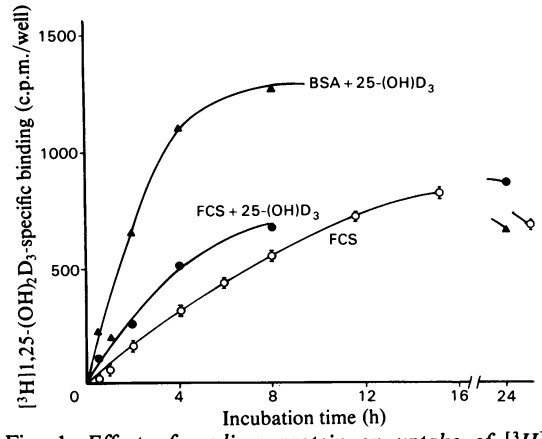

Fig. 1. *Effect of medium protein on uptake of* [^{3}*H*]-*1,25-*$(OH)_2D_3$ *by T47 D cells*

Cells were incubated for various times with [^{3}H]1,25-$(OH)_2D_3$ (70 pM) in the presence of 0.05% bovine serum albumin (BSA) or 5% foetal-calf serum (FCS) with 25-$(OH)D_3$ (25 nM) or with 5% foetal-calf serum without 25-$(OH)D_3$. Specific uptake in c.p.m./well is plotted against time in hours. Values are means of triplicate determinations with S.E.M. less than 5% of means.

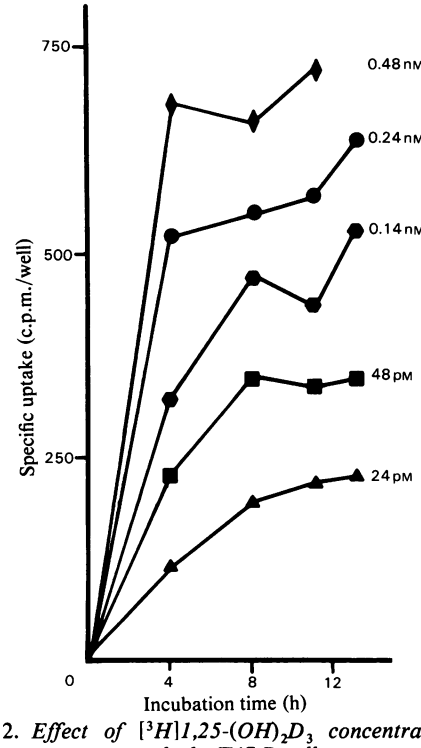

Fig. 2. *Effect of* [^{3}*H*]*1,25-*$(OH)_2D_3$ *concentration on uptake by T47 D cells*

Cells were incubated for various times with various concentrations of [^{3}H]1,25-$(OH)_2D_3$ in the presence of 0.05% BSA. Values are means of triplicate determinations of specific binding with S.E.M. less than 5% of means.

25-(OH)D_3 did not alter the rate of uptake or maximum specific binding reached under these conditions (Fig. 1). However, the addition of high concentrations of various vitamin D metabolites during the incubation in the absence of foetal-calf serum decreased specific binding of 1,25-$(OH)_2D_3$ to extents similar to their biological activity. The potences of various metabolites and analogue relative to 1,25-$(OH)_2D_3$ (arbitrarily 100 units) were 1,24,25-$(OH)_2D_3$ (42), 25-(OH)D_3 (0.5), 24,25-$(OH)_2D_3$ (0.2) and 25,26-$(OH)_2D_3$ (<0.1). Oestradiol, which was added as a further control, had a relative potency of less than 0.1 units. There was no specific uptake of 25-(OH)D_3 at any time up to 6h (results not shown).

The rate of uptake and maximum specific binding reached were both dependent on the concentration of added [^{3}H]1,25-$(OH)_2D_3$ (Fig. 2). Even at the highest concentrations used, uptake of [^{3}H]1,25-$(OH)_2D_3$ was negligible under $\frac{1}{2}$–1h. By contrast the uptake of [^{3}H]oestradiol under identical conditions was complete by 1h and decreased thereafter (E. Sher, J. A. Eisman, J. M. Moseley & T. J. Martin, unpublished work). Analysis of the data from 10–12h of incubation indicated a K_d of 68pM and a receptor number of 11800 sites/cell (Fig. 3).

The rate of egress of radioactivity from pre-incubated cells is shown in Fig. 4. The specifically

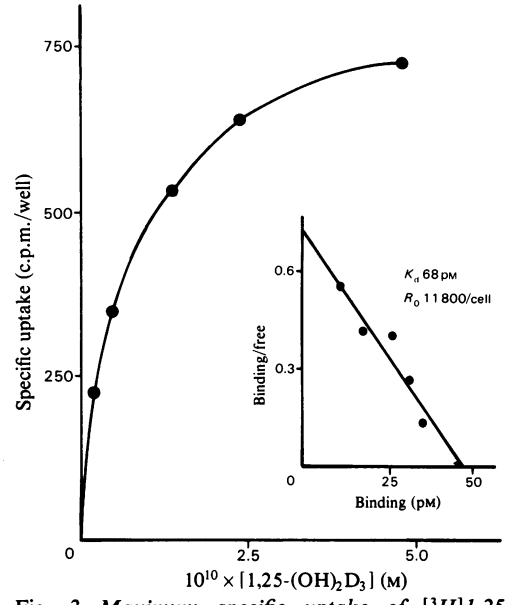

Fig. 3. *Maximum specific uptake of [^{3}H]1,25-$(OH)_2D_3$ by T47 D cells versus concentrations of [^{3}H]1,25-$(OH)_2D_3$*

Values are taken from 10–12h of incubation as shown in Fig. 2. The inset shows Scatchard analysis with line fitted by the least-squares method of linear regression.

Table 1. *Distribution of 1,25-$(OH)_2D_3$ receptor within T47 D cells*

(*a*) Distribution of receptor was determined in 1,25-$(OH)_2D_3$-replete intact cells. Cells were incubated with [^{3}H]1,25-$(OH)_2D_3$ with or without excess unlabelled 1,25-$(OH)_2D_3$, and the specific binding of [^{3}H]1,25-$(OH)_2D_3$ in the various subcellular fractions prepared as described in the Materials and methods section was determined. Results are expressed as c.p.m./fraction per flask and as a percentage of the total specific uptake per flask. (*b*) Distribution of receptor was determined in 1,25-$(OH)_2D_3$-depleted cells. Flasks identical with those used in (*a*) were harvested and fractionated as described above. Specific binding of [^{3}H]1,25-$(OH)_2D_3$ was then assayed in the fractions indicated as described in the Materials and methods section. Data are from incubation for 2h at 25°C and are expressed as c.p.m./fraction per flask and as a percentage of the sum total of specific binding in all fractions assayed. All values are means of at least triplicate determinations of maximum and non-specific binding. Abbreviation: ND, not determined.

	Distribution of receptor			
	(*a*) 1,25-$(OH)_2D_3$-treated		(*b*) 1,25-$(OH)_2D_3$-depleted	
	(c.p.m./fraction)	(% of homogenate value)	(c.p.m./fraction)	(% of total)
Homogenate	17460	(100)	ND	—
Cytosol	1150	7	11600	82
Mitochondria and microsomes	520	3	0	0
Crude nuclei wash	150	1	ND	—
Plasma membranes and intact cells	0	0	ND	—
Purified Nuclei				
0.1M-KCl extract	490	3	2060	15
0.4M-KCl extract	8560	49		
Residue	1190	7	450	3
Total recovered	12060	70	14110	100

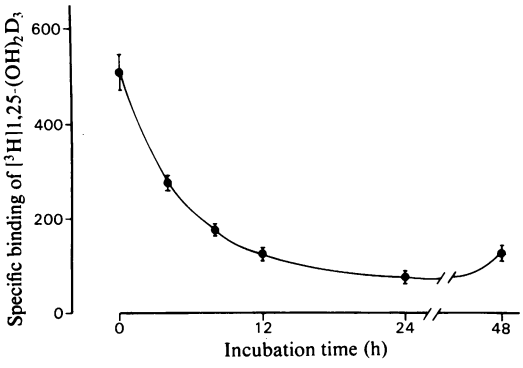

Fig. 4. *Rate of egress of [^{3}H]1,25-$(OH)_2D_3$ from T47 D cells*

Cells were pre-incubated with [^{3}H]1,25-$(OH)_2D_3$ for 16h at 37°C and then changed to 1,25-$(OH)_2D_3$-free medium containing 5% charcoal-treated foetal-calf serum. Values are means of triplicate determinations of specific binding determined after various periods of incubation with S.E.M. less than 5% of means.

bound radioactivity decreased to about 10% of the initial value by 24h.

When T47 D cells were pre-incubated for 16h with [^{3}H]1,25-$(OH)_2D_3$ approximately half of the specifically bound label was found in the purified nuclei and required high-salt concentration to extract it. This was not corrected for the recovery of nuclear DNA, which was 50–60% in parallel experiments; 7% of the recovered specifically bound label remained in the cytosol fraction (Table 1). By contrast in the 1,25-$(OH)_2D_3$-depleted cells about 80% of the specific binding was localized to the cytosol and the remainder was found in the nucleus. Although the absolute amount of specific binding determined in the various fractions by incubation at 4h at 4°C was only about 50% of that determined by incubation at 2h at 25°C, there was no difference in the nucleus/cytosol ratio determined under either condition.

Discussion

The demonstration of specific uptake of 1,25-$(OH)_2D_3$ by the intact T47 D cells in culture indicates that the cytosol receptor described previously is not an artefact of cell disruption. The uptake is specific for 1,25-$(OH)_2D_3$ since the relative potencies of the various vitamin D metabolites and analogues parallels their potency *in vivo*. Furthermore specific uptake of 25-$(OH)D_3$, the immediate precursor of 1,25-$(OH)_2D_3$, was not demonstrable in these cells. The relative potency of 25-$(OH)D_3$ in reducing uptake was decreased in the presence of serum, which is consistent with a decrease in free 25-$(OH)D_3$ concentration. This effect and the reduction of 1,25-$(OH)_2D_3$ uptake in the presence of serum is presumably due to the serum vitamin D-binding protein. However, binding of [^{3}H]1,25-$(OH)_2D_3$ to extracellular or intracellular non-specific binding sites is unlikely to be a major factor in the slow rate of uptake since the addition of excess 25-$(OH)D_3$, which would be expected to occupy at least a proportion of such sites, did not materially alter the rate of uptake. The uptake of 1,25-$(OH)_2D_3$ by the T47 D cells growing in monolayer culture continues for several hours. Similar observations (E. Sher, J. A. Eisman, J. M. Moseley & T. J. Martin, unpublished work) have been made in cloned osteosarcoma cells (Partridge *et al.*, 1980) and intact MCF-7 breast cancer cells. The rate of 1,25-$(OH)_2D_3$ uptake is concentration-dependent as for oestradiol in these cells (E. Sher, J. A. Eisman, J. M. Moseley & T. J. Martin, unpublished work) and in dispersed uterine cells (Muler *et al.*, 1979). Even at concentrations 5-fold higher than 'physiological' levels of 1,25-$(OH)_2D_3$, there is negligible uptake of 1,25-$(OH)_2D_3$ over periods up to 1h. The apparent rate of uptake is considerably slower than one would expect from comparison with cytosol preparations from these cells where equilibrium of 1,25-$(OH)_2D_3$ binding is reached within 1–2h at 25°C. The mechanism of this slow uptake with intact, but not disrupted cells, is not due to extracellular protein binding nor is there any evidence that there are differing classes of binding sites as examined by Scatchard analysis. The K_d and receptor concentration derived in this manner are very similar to those found in the cytosols prepared from disrupted cells (Eisman *et al.*, 1979, 1980*a,b,c*; Martin *et al.*, 1980; Findlay *et al.*, 1980). However, in the latter instance binding equilibrium is reached within 1–2h at 25°C. The rate of egress of ^{3}H from pre-labelled cells is also slow, but is largely complete by 24h. This demonstrates the reversibility of binding and indicates that 24–48h of incubation in charcoal-treated medium should be adequate to effect 1,25-$(OH)_2D_3$ depletion of the cells.

Studies on the localization of the receptor in the [^{3}H]1,25-$(OH)_2D_3$-treated and 1,25-$(OH)_2D_3$-depleted cells are of particular interest. The data indicate that the unoccupied receptor is located in the cytosol even using the low-ionic-strength lysis buffer. There was only about 15% of the receptor located in the purified nuclei from the 1,25-$(OH)_2D_3$-depleted cells. Furthermore this receptor did seem to be unoccupied as there was a similar cytosol/nuclear ratio, whether the fractions were assayed for specific binding under exchange (2h at 25°C) or non-exchange (4h at 4°C) conditions. Clearly this distribution differs from that claimed for the chicken intestinal receptor, all of which has been reported to

associate with purified nuclei under low-ionic-strength conditions even in the unoccupied state (Walters *et al.*, 1980). Nuclear translocation of the receptor after pre-incubation with [^{3}H]1,25-$(OH)_2D_3$ is indicated by the fact that the receptor–hormone complex is largely localized to the purified nuclei and is resistant to extraction by 0.1 M-KCl but can be extracted with 0.4 M-KCl.

In summary, the uptake of [^{3}H]1,25-$(OH)_2D_3$ by the T47 D cells in culture is specific for 1,25-$(OH)_2D_3$. The binding affinity and receptor concentration are similar to those previously reported for the breast cancer cells and to the reported higher-affinity binding of chicken bone receptor (Mellon & DeLuca, 1980). The rate of uptake is very slow by comparison with that of other steroid hormones, e.g. oestradiol, and the explanation for this delay deserves further study. Finally there is clear evidence of translocation of the receptor from the cytosol to the nucleus. These findings support the hypothesis that the 1,25-$(OH)_2D_3$ receptor that is present in cultured breast cancer cells, in normal functional rabbit breast tissue and in primary breast cancers, may represent the first component of a 1,25-$(OH)_2D_3$-dependent function, probably that of mineral transport. This possibility is supported by work (Fry *et al.*, 1980) suggesting stimulation by 1,25-$(OH)_2D_3$ of Ca^{2+} transport into rat breast milk. It seems reasonable to suggest that these cultured breast cancer cells may provide a valuable model *in vitro* for the further study of the early biochemical events in 1,25-$(OH)_2D_3$ action in its target organs.

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